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Latent Membrane Protein 1 Deletion Mutants Accumulate in Reed-Sternberg Cells of Human Immunodeficiency Virus-Related Hodgkin's Lymphoma

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The origin and biological significance of deletions at the 3′ end of the Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP-1) gene are still controversial. We herein demonstrate that LMP-1 deletion mutants are highly associated with human immunodeficiency virus-related Hodgkin's lymphoma (HIV-HL) of Italian patients (29 of 31 cases; 93.5%), a phenomenon that is not due to a peculiar distribution of EBV strains in this area. In fact, although HIV-HL patients are infected by multiple EBV variants, we demonstrate that LMP-1 deletion mutants preferentially accumulate within neoplastic tissues. Subcloning and sequencing of the 3′ LMP-1 ends of two HIV-HL genes in which both variants were present showed the presence of molecular signatures suggestive of a likely derivation of the LMP-1 deletion mutant from a nondeletion ancestor. This phenomenon likely occurs within tumor cells in vivo, as shown by the detection of both LMP-1 variants in single microdissected Reed-Sternberg cells, and may at least in part explain the high prevalence of LMP-1 deletions associated with HIV-HL.

Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus asymptomatically carried by more than 95% of the human population. The virus has been implicated in the pathogenesis of undifferentiated nasopharyngeal carcinoma, endemic Burkitt's lymphoma, posttransplant lymphoproliferative disorders, and classical Hodgkin's lymphoma (HL) (17). While only a fraction of HL from the general population is EBV associated, human immunodeficiency virus-related HL (HIV-HL) is pathogenetically linked to EBV in 80 to 100% of cases (1, 8, 14, 17, 26, 29). Besides EBV-encoded RNAs, EBNA-1, and latent membrane protein 2 (LMP-2), Hodgkin and Reed-Sternberg cells (H-RSCs) in these cases invariably show a strong expression of the oncogenic LMP-1 protein (1, 4, 8, 17, 26), an integral membrane protein of 386 amino acids able to exert pleiotropic effects essential for EBV-induced B-cell transformation (11, 17). Recent biochemical studies have shown that LMP-1 activates the NF-κB and AP-1 transcription factors and triggers the JAK/STAT pathway (11) by recruiting signal-transducing molecules at docking sites in its C-terminal region. Interestingly, this region is highly heterogeneous across diverse isolates, showing clustered point mutations, insertions, and in-frame 30- or 69-bp deletions (3, 17, 30). A high prevalence of the 30-bp LMP-1 deletion mutants was reported in undifferentiated nasopharyngeal carcinoma (6, 15), peripheral T-cell lymphomas (22), posttransplant lymphoproliferative disorders (25), and, particularly, in aggressive HIV-unrelated HL and HIV-HL (2, 9, 10, 19, 22), suggesting that these LMP-1 deletion mutants may have increased transforming potential. Accordingly, LMP-1 deletion forms display enhanced tumorigenicity and reduced immunogenicity compared with those of LMP-1 of strain B95.8, in animal and in vitro models (6, 16, 27, 28). Nevertheless, the real pathogenetic significance of deletions and/or mutations within the 3′ end of the LMP-1 gene still awaits a conclusive demonstration. Moreover, it is not clear whether the high prevalence of LMP-1 deletion variants detected in some EBV-related disorders merely reflects the predominance of these variants in some geographic areas or is rather due to peculiar virus-host interactions leading to enhanced production and/or selection of LMP-1 deletion mutants. To address this issue, we have reassessed the association between LMP-1 deletion mutants and HIV-HL by characterizing the 3′ LMP-1 variants infecting a large group of Italian HIV-HL patients in comparison with those detected in reactive lymphadenopathies from HIV-seropositive patients of the same geographic area. Moreover, the EBV variants concurrently present at diverse anatomic sites in HIV-HL patients were also characterized by multilocus analysis. Molecular analyses were performed on 32 tumor biopsy samples obtained from 31 HIV-HL patients identified by searching the files of the Division of Pathology of the Centro di Riferimento Oncologico, Istituto di Ricovero e Cura a Carattere Scientifico,

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National Cancer Institute (Aviano, Italy), the S. Raffaele Scientific Institute (Milan, Italy), and the Institute of Biomedical Sciences (Milan, Italy). Association with EBV was verified in all cases by EBV-encoded RNA in situ hybridization and immunohistochemistry for the LMP-1 expression, as described (4, 5). LMP-1 variants were characterized by use of a touchdown PCR that encompassed the 33-bp repeat and the deletion region of the LMP-1 gene, allowing the concurrent evaluation of two polymorphic loci within the 3′ region of the gene. Genomic DNA was extracted from snap-frozen or formalin-fixed tissue by use of a silica gel spin cartridge after proteinase K digestion. The carboxy terminus of the LMP-1 gene (nucleotide [nt] positions 168209 to 168649) was amplified by a first round of PCR (primer 1s, 5′ CCA CCT GCT CGT GAG TGG AGC 3′; primer 1as, 5′ CCA CCG GAA CCA GAA GAA CCC 3′) and used as a common template for the amplification of 33-bp repeats and either 30- or 69-bp deletion regions by a second round of nested PCR. Amplification of the internal 33-bp repeat region was performed using the first-round sense primer 1s and a second-round antisense primer (primer 2as, 5′ GGC CCT CCA TCA TTT CCA GCA 3′); a second primer (primer 2s, 5′ TGC TGG AAA TGA TGG AGG CCC 3′), complementary to the region recognized by primer 2as, and the first-round antisense primer 1as were used to amplify the deletion region. Primers were chosen within regions of the LMP-1 carboxy terminus showing a limited degree of sequence heterogeneity among natural isolates (30). Amplification reaction mixtures contained 2 to 5 μl of DNA, 1× AmpliTaq Gold PCR Master mix II (Perkin-Elmer Life Sciences, Boston, Mass.), 1.5 mM $MgCl_2$, and a 200 nM concentration of each of the specific primers in a total volume of 50 μl. Both rounds were performed with a "hot-start" procedure by using AmpliTaq Gold (Perkin-Elmer Life Sciences), followed by a touchdown program (94°C for 30 s, 64°C for 45 s, decreasing by 1°C every second cycle down to 55°C, 72°C for 45 s, and a further 15 cycles at an annealing temperature of 55°C). PCR products were separated by agarose gel electrophoresis, and the sizes of the fragments were assessed by image analysis (Image Station 440CF; Kodak Digital Science, NEN Life Science Products, Boston, Mass.). Both the presence of 30- and 69-bp deletions and the number of 33-bp repeats were predicted by the fragment size as calculated by the software. This approach proved to be highly sensitive and specific in detecting different LMP-1 variants from natural EBV isolates, even when present in small amounts (1 to 5 molecules per reaction [data not shown]). In particular, compared with a low-constant-annealing-temperature PCR, higher rates of LMP-1 detection of both single and multiple coinfecting variants were observed in different series (not shown), indicating that this PCR protocol may overcome possible obstacles to PCR amplification related to the sequence heterogeneity of this region.

LMP-1 deletion mutants are significantly more prevalent in HIV-HL than in HIV-related reactive lymphadenopathies. Twenty-nine of the 31 informative HIV-HL cases (93.5%) carried the 30-bp (28 cases) or the 69-bp (1 case) LMP-1 deletion variants, confirming also in this series the high prevalence of LMP-1 deletions previously found in patients with this disease (2, 8, 23) (Table 1). In 17 of the 28 cases carrying the 30-bp deletion (60.7%), faint bands corresponding to full-length LMP-1 variants were also detected, although only at low dilutions of template DNA, indicating a likely derivation from EBV-positive bystander B cells. Interestingly, in two cases (HL21 and HL31), both deletion mutants and full-length fragments were invariably found. In one of the two cases carrying a predominant full-length LMP-1 (HL20), a minor variant with the 30-bp deletion was also detected. We have also verified that the high prevalence of LMP-1 deletions detected in HIV-HL cases could also be found in benign lymphoproliferations arising in HIV-infected patients with similar sociodemographic characteristics (sex, age, ethnicity, geographic origin, and date of diagnosis). Analysis of 27 cases of HIV-related reactive lymphadenopathy showed a prevalence of LMP-1 deletion variants significantly lower than that in cases of HIV-HL (14 of 27, 51.9%; $P < 0.001$) but similar to that observed in cases of HIV-unrelated HL (40%) and in normal peripheral blood mononuclear cells from both HIV-infected (54.1%) and HIV-seronegative (55.6%) Italian individuals (10). These findings indicate that the high prevalence of LMP-1 deletions observed in HIV-HL cases does not reflect the distribution of LMP-1 variants in this geographic area. Moreover, our results also rule out the possibility that the reactive lymphoproliferative process per se might increase the production or local recruitment of LMP-1 deletion mutants in HIV-infected individuals.

Patients with HIV-HL are infected by multiple EBV variants, but LMP-1 deletion mutants accumulate within neoplastic tissues. We also sought to determine whether the high proportion of deletion mutants found in HIV-HL patients simply reflects the presence of an LMP-1 deletion mutant strain predominantly infecting these patients or is rather due to a disease-specific segregation of deletion variants within HL tissues. To this end, we characterized the LMP-1 variants in neoplastic and uninvolved tissues ($n = 14$) from 10 HIV-HL patients, selected on the basis of tissue availability (Table 2). Two patients (HL17 and HL30) carried a 30-bp LMP-1 deletion variant in both involved and uninvolved tissues (Table 2). Notably, five patients with 30-bp deletions in the tumor biopsy samples (HL22, HL23, HL27, HL28, and HL29) carried full-length LMP-1 in all uninvolved tissues investigated. Two patients with full-length LMP-1 in the HL biopsy samples (HL18 and HL20) carried a 30-bp LMP-1 deletion variant in three of four uninvolved samples. Finally, patient HL21 showed codominance of a 69-bp deletion mutant and full-length LMP-1 in both the HL biopsy sample and the cutaneous Kaposi's sarcoma lesion sample (Table 2). These findings indicate that the majority of HIV patients with HL in this study harbored a mixed population of both full-length and deletion variants of LMP-1, but, in 50% of these patients, the LMP-1 deletion variant was predominantly found only in HL tissue.

Results of the characterization of EBV variants detected in HIV-HL biopsy samples are shown in Table 1. Amplification of the LMP-1 fragment encompassing the 33-bp repeat region was successfully performed for 26 HIV-HL cases, demonstrating product sizes compatible with a repeat number ranging from 4 to 7.5 (confirmed by direct sequencing of gel-purified PCR products in a fraction of cases [data not shown]). No association was found between the number of 33-bp repeats and the presence of the 30-bp deletion (Table 1). A single 33-bp size variant was detected in 18 cases, whereas an additional band was found in eight biopsy samples. In seven of the

TABLE 1. Characterization of EBV variants in HIV-HL biopsy samples[a]

Patient no.	Anatomic site	EBV type	LMP-1 variant[b]	No. of 33-bp repeats[c]
HL1	LN	2	30-bp del.	ND
HL2	LN	1	30-bp del.	ND
HL3	LN	2	30-bp del.*	4/5
HL4	LN	1	30-bp del.	5
HL5	LN	2	30-bp del.*	4
HL6	LN	1	30-bp del.*	7
HL7	LN	1	30-bp del.*	6.5
HL8	LN	1	30-bp del.	4
HL9	LN	1	30-bp del.	5/6.5
HL10	LN	ND	30-bp del.*	6.5/7
HL11	LN	ND	30-bp del.	4
HL12	LN	ND	30-bp del.*	
HL13	LN	1	30-bp del.*	
HL14	LN	2	30-bp del.	4
HL15	LN	1	30-bp del.*	4
HL16	LN	2	30-bp del.*	5
HL17	LN	1[d]	30-bp del.*	7
	Liver	1	30-bp del.*	7
HL18	LN	1	Full-length	4
HL19	LN	1/2	30-bp del.*	4/5
HL20	LN	1/2	Full-length**	5/7
HL21	LN	2	69-bp del. plus full-length	6.5
HL22	LN	1/2	30-bp del.	5
HL23	Spleen	ND	30-bp del.*	6.5/7.5
HL24	LN	ND	30-bp del.*	
HL25	LN	1	30-bp del.	4
HL26	LN	ND	30-bp del.*	7
HL27	LN	ND	30-bp del.*	6.5/7
HL28	Bone marrow	1	30-bp del.*	5
HL29	LN	2	30-bp del.	6.5/7
HL30	LN	1[d]	30-bp del.*	4
HL31	LN	2[e]	30-bp del. plus full-length	5

[a] Abbreviations: LN, lymph node; ND, not determined; del., deletion.
[b] *, a less-represented full-length LMP-1 was also detected. **, a less-represented 30-bp deletion mutant was also detected.
[c] The predominant 33-bp repeat variant is underlined.
[d] Case with a T-to-G change at nt 1211 (strain B95.8, accession no. K03333).
[e] Case with a G-to-T change at nt 1318 (isolate AG876, accession no. K03333) of the EBNA-2 U2-IR2 domain (accession no. K03333).

eight biopsy samples, one of the two 33-bp variants was less represented (as shown by serial dilutions of template DNA), consistent with a possible derivation from EBV-positive bystander B cells. Notably, 11 of the 18 patients (66.7%) with a single 33-bp size variant carried both deletion mutants and full-length LMP-1 in the same biopsy sample (Table 1). Furthermore, EBV typing (23) carried out for 8 of these 11 HIV-HL biopsy samples showed a single EBV subtype in each of these cases (six cases with EBV type 1 and two cases with EBV type 2). Moreover, a monoclonal EBV episome was also demonstrated in six of these cases by analysis of the terminal repeat region (data not shown). The presence of a single EBV strain was confirmed in 5 of the HIV-HL biopsy samples with a single virus subtype and one 33-bp size variant but showing both full-length and deletion variants of LMP-1 (HL7, HL17, HL21, HL30, and HL31). In fact, subcloning and sequencing of the EBNA-2 U2-IR2 domain (nt 48170 to 48339 of the strain B95.8 genome) showed that all subclones (at least 10) from each of these cases had identical EBNA-2 sequences. Although this EBNA-2 region is not highly polymorphic, strain-specific polymorphisms were detected in three of these cases (Table 1). Taken together, the results of our multilocus analysis are consistent with the possibility that, in HIV-HL patients, LMP-1 deletion variants may originate from endogenous EBV strains carrying full-length LMP-1.

H-RSCs of HIV-HL harbor both deletion and full-length variants of LMP-1, suggesting a common origin of these variants. To identify molecular signatures supporting the possible derivation of the deletion variant from an ancestor EBV strain with a full-length LMP-1, the C terminus of the LMP-1 gene, comprising the 33-bp repeat and the deletion regions (nt 168209 to 168649), was subcloned into pGEM-T vector from five HIV-HL biopsy samples (HL5, HL7, HL21, HL30, and HL31). Plasmid DNA was purified using a QIAprep 8 Turbo Miniprep kit (QIAGEN, Studio City, Calif.) and sequenced on both DNA strands by using SP6 and T7 primers and the dye terminator protocol on an ABI 310 genetic analyzer (Perkin-Elmer Corp., Foster City, Calif.). Only subclones carrying deletions of LMP-1 could be obtained in patients HL5, HL7, and HL30, consistent with the abundance of this variant in these HL biopsy samples. Conversely, subclones carrying full-length or deletion variants of LMP-1 were detected in both cases HL21 and HL31. Notably, subclones of each of these dual-variant cases had the same number of internal 33-bp repeats and shared some hallmark mutations (Fig. 1), suggesting their likely derivation from the clonal EBV infecting H-RSCs.

TABLE 2. Comparative genotypic analysis of LMP-1 variants in neoplastic and nonneoplastic tissues from the same patients with HIV-HL[a]

Patient no.	Anatomic site	LMP-1 variant[b]	No. of 33-bp repeats[c]
HL17	Lymph node (involved)	30-bp del.*	7
	Spleen	30-bp del.*	7
HL18	Lymph node (involved)	Full-length	4
	Bronchus	30-bp del.*	
	Lymph node	30-bp del.*	4/6.5
	Spleen	Full-length**	4
HL20	Lymph node (involved)	Full-length**	5/7
	Uterine cervix	30-bp del.*	7
HL21	Lymph node (involved)	69-bp del. plus full-length	6.5
	Skin (KS lesion)	69-bp del. plus full-length	6.5
HL22	Lymph node (involved)	30-bp del.*	5
	Lung	Full-length	4/5
HL23	Spleen (involved)	30-bp del.*	6.5/7.5
	Lung	Full-length**	6.5/7.5
HL27	Lymph node (involved)	30-bp del.*	6.5/7
	Liver	Full-length**	6.5
	Bronchoalveolar lavage sample	Full-length**	7
	Liver	Full-length**	7
HL28	Bone marrow (involved)	30-bp del.*	5
	Bone marrow	Full-length	5/7
	Gastric mucosa	Full-length	5/7
HL29	Lymph node (involved)	30-bp del.	6.5/7
	Gastric mucosa	Full-length	7
	Bone marrow	Full-length	7
HL30	Lymph node (involved)	30-bp del.	4
	Bronchoalveolar lavage sample	30-bp del.	

[a] Abbreviations: KS, Kaposi's sarcoma; del., deletion.
[b] *, a less-represented full-length LMP-1 was also detected. **, a less-represented 30-bp deletion mutant was also detected.
[c] The predominant 33-bp repeat variant is underlined. Two numbers separated by a slash indicate the presence in a tumor biopsy specimen of two different variants identified by a distinct number of 33-bp repeats. Each number is the relative number of 33-bp repeats that was assessed as described in the text.

To obtain further evidence that H-RSCs of HIV-HL may harbor clonally related EBV variants with both deletion and full-length variants of LMP-1, a similar analysis was carried out with microdissected H-RSCs from cases HL21 and HL31 (Fig. 2). DNA obtained from 20 $CD30^+$ H-RSCs isolated by laser microdissection was preamplified by the improved primer extension preamplification protocol (7) and used as a template for PCR amplification. For both cases, the analysis consistently detected both deletion and full-length variants of LMP-1, carrying the same number of 33-bp repeats. Moreover, sequencing of LMP-1 subclones derived from pooled H-RSCs confirmed the presence of shared mutations (Fig. 1). Single-cell analysis performed with samples from patients HL21 and HL31 by using 10 H-RSCs/sample disclosed the presence of both deletion and nondeletion variants in three and two individual H-RSCs, respectively (data not shown), ruling out the possibility that different H-RSCs from the same specimen might harbor different EBV variants and further strengthening the hypothesis of a common origin of the two LMP-1 variants.

The results of the present study demonstrate that the high prevalence of LMP-1 deletion mutants detected in HIV-HL is independent of the geographic distribution of EBV strains and provide further evidence indicating that these LMP-1 deletion variants are strictly associated with HIV-HL of the Italian population. EBV genotypes with LMP-1 deletions may infect humans as independent variants, or, alternatively, these EBV variants may arise as a result of inter- or intrastrain recombination during productive EBV replication (30). Considering that in both HIV-seronegative and HIV-infected Italian individuals the prevalence of LMP-1 deletions is near 50%, we can in good faith assume that, in about half of the patients with HIV-HL, the presence of the deletion variant is likely due to a de novo infection of H-RSC precursors by an EBV strain carrying an LMP-1 deletion. Notwithstanding, we herein provide evidence suggesting the possibility that, at least in a proportion of HIV-HL cases, the deletion variant may also derive from an ancestor EBV with full-length LMP-1. This conclusion is particularly supported by the detection of both LMP-1 variants in microdissected H-RSCs from two cases, suggesting that the LMP-1 deletion mutant probably originates within tumor cells in vivo. These results are in keeping with those recently reported by Kim et al. (18), who detected a mixed pattern of LMP-1 variants in single H-RSCs from two cases of HIV-unrelated classical HL. Considering that the EBV infecting

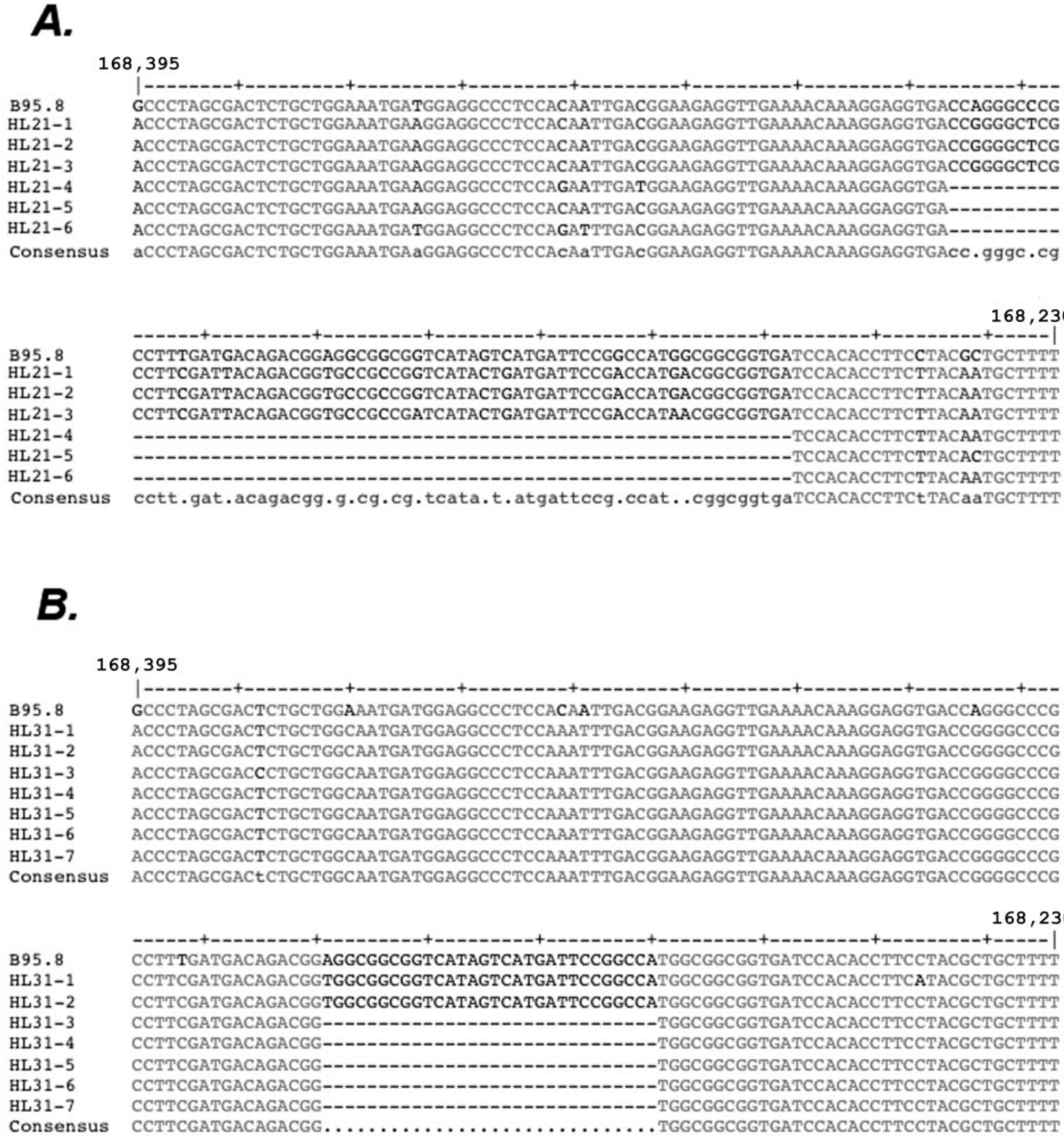

FIG. 1. Alignment of DNA sequences from LMP-1 3′ subclones derived from two HL patients carrying both LMP-1 deletion mutants and full-length LMP-1 in the neoplastic tissue. Sequence analysis of LMP-1 subclones obtained from pooled microdissected H-RSCs from patients HL21 (A) and HL31 (B), showing the presence of LMP-1 deletion and nondeletion variants together with shared hallmark mutations. Differences in the nucleotide sequences of the various LMP-1 subclones are indicated in bold.

H-RSCs is strictly latent, it is unlikely that in these cells LMP-1 deletions might originate from intra- or interstrain recombination events during virus replication, in contrast to what was observed in patients with oral hairy leukoplakia (31). Our findings rather support the hypothesis that deletion variants can be the result of the progressive accumulation of mutational events at the C-terminal LMP-1 hot spot from a single virus strain occurring during latent infection. Although further studies are required to define the mechanisms underlying the generation of LMP-1 deletions within H-RSCs, the strict association of these variants with HIV-HL suggests a role for disease-related phenomena. As previously hypothesized (3), the generation of LMP-1 deletions could be favored by processes, such as somatic hypermutation or immunoglobulin class switching, that are associated with the persistent germinal-center activity that characterizes HIV-infected individuals. In this scenario, the high prevalence of LMP-1 deletions observed in patients with HIV-HL could be due to a possible selective advantage of variants carrying the deletion and/or specific mutations within the LMP-1 C terminus, as suggested by some functional data (6, 16, 27, 28). Nev-

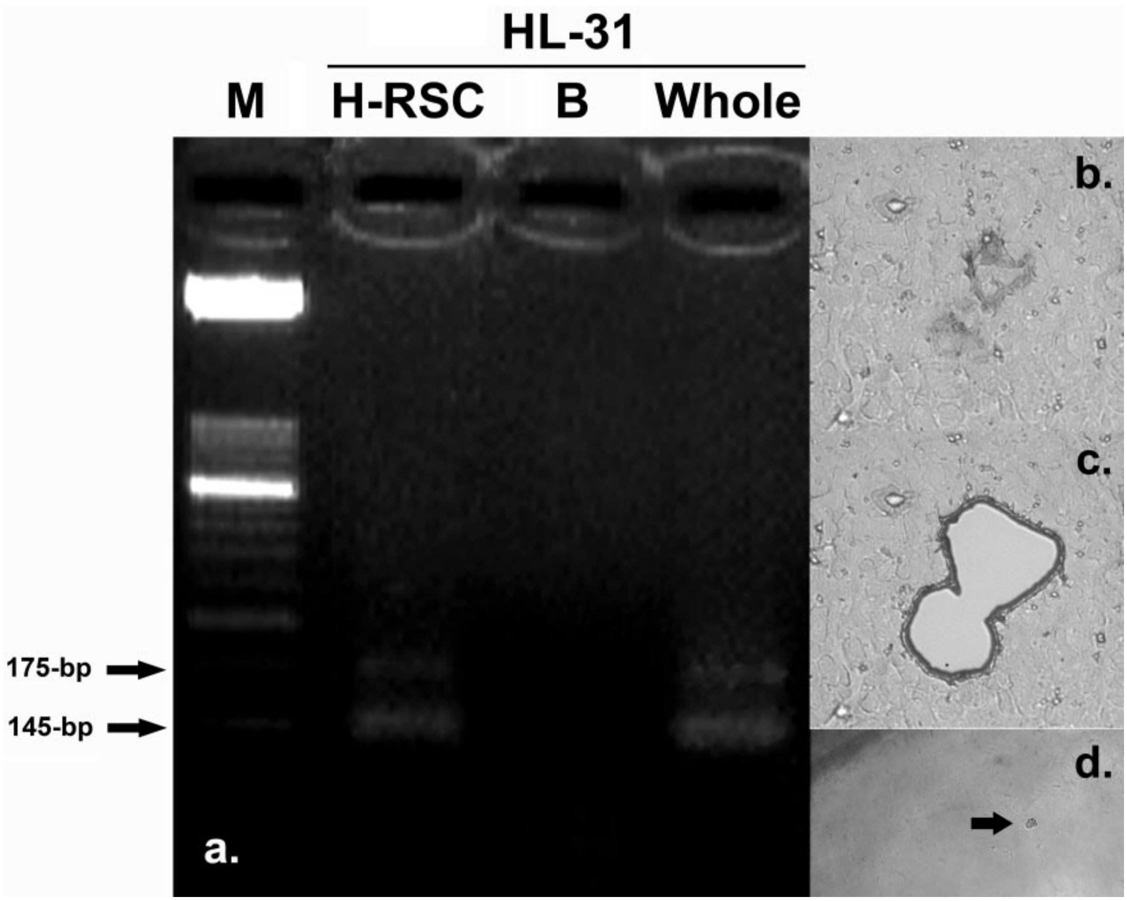

FIG. 2. Microdissection of H-RSCs and analysis of the LMP-1 3′ end of case HL31. (a) Gel electrophoresis demonstrating that both the 30-bp deletion and full-length LMP-1 variants were detected in whole tissue and H-RSCs isolated by microdissection from case HL31. Lanes: M, DNA molecular weight marker, 50-bp ladder (Roche Diagnostics GmbH, Mannheim, Germany); B, negative control, containing nontemplate DNA from PCR master mix. (b) Identification of CD30$^+$ H-RSCs by immunohistochemistry. (c) H-RSCs selectively laser captured and successfully collected in the cap of a PCR tube. (d) One laser-captured H-RSC, indicated by an arrow.

ertheless, it remains to be determined whether still unidentified host- and/or disease-related factors may have a role in favoring the generation of LMP-1 deletion variants in patients with HIV-HL. Elucidation of this issue will allow a better understanding of the pathogenesis of this malignancy, giving useful insights on the possible roles played by different LMP-1 variants.

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